Synopsis

Proteomic approaches to study glioma development, progression and therapy

Astrocytoma, the tumor of astrocytic origin, accounts for about 60 % of the primary brain tumors. As per World Health Organization grading system, astrocytoma is classified as circumscribed astrocytoma (Grade I; pilocytic astrocytoma) and diffusely infiltrating astrocytoma. Grade I tumor is biologically benign and can be cured by surgical resection of the tumor. The diffusely infiltrating astrocytoma is further subclassified into grade II/diffuse astrocytoma (DA), grade III/anaplastic astrocytoma (AA) and grade IV/glioblastoma (GBM). Aggressiveness of the disease increases as the tumor progresses from lower grade to higher grade. In particular, GBMs are the most malignant and aggressive human cancers. For a newly diagnosed GBM patient, the current treatment option is surgical resection of the tumor followed by radiation and temozolomide therapy. Despite the treatment is multimodal (surgery+radiation+temozolomide) the median survival of GBM patients remain very low at 14.6 months. Although numerous markers with potential utility in prognosis and treatment of GBMs have been reported, they are yet to be translated into clinical utility. Our knowledge of understanding the complete biology of GBMs needs further comprehensive studies towards the identification of markers with potential utility to prognose/treat the GBM patients efficiently. Therefore, with an immense need to develop new biomarkers/therapeutic strategies in order to improve the diagnosis, prognosis and existing treatment of the GBM, the current work is designed to study the following aspects on glioma.

1. Identification of glioblastoma-specific serum biomarker(s)

2. Proteomic analysis of temozolomide induced changes in glioblastoma

1. Identification of glioblastoma-specific serum biomarker(s)

Biomarker refers to a molecule whose presence reflects a particular disease state or severity of a disease or some other physiological condition of an organism. In cancer, biomarkers have been extensively in use for disease diagnosis, prognosis and monitoring the treatment. To efficiently use the biomarkers for cancer clinical management, the source of the biomarker must be obtained through less invasive or noninvasive method(s). GBM is an aggressive intrinsic primary brain tumor. Therefore, identification of the biomarker(s) from cerebrospinal fluid (CSF) will appropriately reveal the presence of a disease state in the brain. But obtaining CSF from patients by lumbar puncture is a highly invasive method. The next appropriate source for mining the biomarker(s) appears to be serum. Serum is obtained by simple non-invasive method. Remarkably, the serum components are present in a highly dynamic range, it essentially reflects the normal or diseased status of an individual.

In the present study, we used preoperative serum samples from GBM patients to identify biomarkers using two dimensional gel electrophoresis (2-DE) and MALDI-TOF mass spectrometry. 2-DE analysis of sera from normal healthy individuals (N=6) and GBM (N=14) patients was carried out. ImageMaster 2D Platinum version 7.0 software was used for the quantitative evaluation of differentially expressed proteins. Compared to normal individual serum, there were several spots found differently expressed in the glioblastoma sera. However, statistical analysis revealed eight spots (spots 1 to 8) to be up-regulated and two spots (spots 9 and 10) down-regulated significantly ($p<0.05$) in glioblastoma. The spots 1 to 6 had a calculated molecular weight of 20.26 kDa and their pI ranged from 5.5 to 7.1. Based on the existing literature and human serum 2-DE databases available, these six spots were predicted to be haptoglobin alpha2. Accordingly, MALDI-TOF analysis of spots 2, 3

and 4 identified these spots as haptoglobin2 alpha (Hp2-alpha). Subsequently, using haptoglobin specific antibody, we have also confirmed that these six spots are isoforms of Hp2-alpha. Spot 7 and spot 8 are identified as MHC class I antigen, Angiopoietin 2 isoform b precursor respectively. Spot 9 and spot 10 corresponded to Apolipoprotein A-1. Haptoglobin is a acute phase serum glycoprotein synthesized and secreted into blood stream by hepatocytes. The haptoglobin molecule is a tetrameric protein with two α/β dimmers. The α subunit exists as $\alpha 1$ (unduplicated, 10 kDa) or $\alpha 2$ (duplicated, 19 kDa) but β subunits (undergo heavy glycosylation) are identical. The well known function of haptoglobin is that it binds free hemoglobin and it prevents iron loss and renal damage.

It was very prominent from our serum 2-DE analysis that Hp2-alpha isoforms were significantly elevated in the GBM patient's sera. Therefore, we investigated in detail the GBM specific up-regulation and functional consequence of haptoglobin in astrocytoma. Further, quantitation of serum haptoglobin on large cohort of samples by western blot analysis and ELISA in normal controls and patients with different grades of astrocytoma confirmed that haptoglobin is indeed significantly upregulated in GBM patient's sera. We then questioned whether the elevated serum haptoglobin is derived from GBM. To address this possibility, we monitored the expression of haptoglobin mRNA and protein between normal controls and different grades of astrocytoma including GBM. For the first time, we provide evidence that increased haptoglobin mRNA and protein expression is observed in astrocytoma and the expression level is significantly higher in GBMs ($p=<0.0001$ and <0.0001 respectively). Using receiver operating characteristic (ROC) curve, we have also demonstrated that GBM-specific elevation of Hp can differentiate GBM from normals and lower grades of astrocytoma. Further, we studied the putative role of haptoglobin in

astrocytoma. Overexpression of Hp either by stable integration of Hp cDNA or exogenous addition of purified Hp to immortalized astrocytes resulted in increased cell migration. RNAi-mediated silencing of Hp in glioma cells decreased cell migration. Further, we demonstrate that both human glioma and mouse melanoma cells overexpressing Hp showed increased tumor growth.

2. Proteomic analysis of temozolomide induced changes in glioblastoma

Glioblastoma (GBM), the grade IV glioma, is highly malignant brain tumor. GBMs are treated by first surgical resection of the tumor followed by radiation and temozolomide (TMZ) therapy. Addition of TMZ, a DNA alkylating agent, to postoperative radiotherapy has been shown to improve median survival and two-year survival significantly compared with postoperative radiotherapy alone.

Although, DNA repair enzyme alkyl O^6-alkylguanine-DNA alkyl transferase (MGMT), deficient mismatch repair (MMR) system, Poly ADP-ribose polymerase (PARP) have been shown to determine the sensitivity of TMZ to GBM, the mechanism of action of TMZ is not completely understood yet. Thorough understanding of the TMZ induced alterations in protein profiles of GBM may potentially lead to identification of new therapeutic strategies for treating the GBM tumors. In the present study we employed 2-Dimensional Differential Gel Electrophoresis (2-D DIGE) approach to identify and quantify the TMZ induced changes in the expression of proteins in U251, a glioma derived cell line. We have analyzed the protein profiles of DMSO or TMZ treated U251 cells for 24 h, 48 h and 72 h and the biological variation analysis (BVA) was performed to identify the significantly differentially expressed proteins. Temporal analysis of TMZ induced protein spots revealed a significant (I-ANOVA, $p<=0.05$) change in the expression of 95 protein

spots in which 59 protein spots showed up-regulation and 36 protein spots showed down-regulation. Further, we studied in detail about four upregulated and one downregulated protein spots in detail. The identities of these five proteins are confirmed by MALDI-TOF analysis and mascot database search as 1. glutathione synthetase (GSS), 2. breast carcinoma amplified sequence-1 (BCAS1), 3. interleukin-1 receptor associated kinase- 4 (IRAK4), 4. aspartyl t-RNA synthetase (DARS) and 5. optineurin (OPTN). Validation of the five protein expression upon TMZ treatment was further confirmed by RT-qPCR and western blot analysis in multiple glioma cell lines

We presumed that TMZ upregulated proteins may play role in determining sensitivity of glioma cells to TMZ. To address this question, each of these four genes were downregulated by siRNA approach and we studied the influence of inhibition of expression of these proteins on TMZ sensitivity of glioma cells. Downregulation of DARS, GSS, and BCAS1 did not affect the TMZ sensitivity, whereas the downregulation of IRAK4 was found to significantly ($p<0.05$) make U251 cells resistant to TMZ. Similarly, downregulation of IRAK4 made U138 and LN229 glioma cells significantly resistant to TMZ ($p<0.05$). These set of experiments provided evidence that TMZ-induced IRKA4 is essential to enhance the glioma sensitivity to TMZ treatment. High levels of induced IRAK4 upon TMZ treatment resulted in IRAK1 downregulation and inhibition of NFkB pathway. Further, we show that TMZ treatment in IRAK4 silenced cells failed to inhibit NFkB dependent reporter activity. Thus, we report here the identification of several TMZ modulated proteins and discovered an important novel role for IRAK4 in inhibition of TLR signaling and NFkB pathway in determining TMZ sensitivity of glioma cells.